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A KINETIC STUDY OF BACTERIOCHLOROPHYLL PHEOPHYTINIZATION IN THE PROTEIN COMPLEX FROM A GREEN PHOTOSYNTHETIC BACTERIUM

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SUMMARY

The rate of magnesium removal from bacteriochlorophyll in a chlorophyll–protein complex containing 20 chlorophyll molecules was directly proportional to $[H^+]$. HCl first converted the native blue-green complex to a blue intermediate (I 790) in which some bacteriochlorophyll molecules were exposed to the solvent. The spectrum of the intermediate was determined from the ratio of the initial to the final absorbance values recorded in the kinetic runs. The course of the reaction was then followed in a spectrophotometer by observing either the absorbance decrease at 783 mμ or the absorbance increase at 535 mμ. The difference in the apparent rates observed at the two wavelengths suggested that a second intermediate (I 770) was formed from the first intermediate. An increase in ionic strength decreased the rate of pheophytinization and accentuated the difference between 783-mμ and 535-mμ data. The kinetic data at several values of $[H^+]$, ionic strength and temperature showed that the 20 molecules of bacteriochlorophyll in the protein differ with respect to reactivity towards H^+. The apparent activation energy increased with the course of the reaction and approached the value for chlorophylls in solution. The quantitative differences between the present study and previous observations of the pheophytinization of chlorophylls dissolved in polar solvents are attributed to the protein environment of bacteriochlorophyll in the complex.

INTRODUCTION

The kinetics of pheophytinization of bacteriochlorophyll (Bchl) in an acetone–water mixture have been investigated by PAKSHINA AND KRASNOVSKII[1]. The reaction follows first-order kinetics with respect to Bchl in 0.02 M oxalic acid (pH 2.2).

In the course of a study of a Bchl–protein complex[2,3] isolated from the green photosynthetic bacterium *Chloropseudomonas ethylicum*, a curious feature was noticed

Abbreviations: Bchl, bacteriochlorophyll; Bph, bacteriopheophytin.

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in the changes induced in the absorption spectrum of the complex in acidic media. In the pH interval 1.5 to 3.0, Bchl in the complex was converted to bacteriopheophytin (Bph) and only those two species could be identified in the reaction mixture[4]. Although the rates of pheophytinization of chlorophylls *a* and *b* in solution are proportional to the first power of H^+ concentration[5], the rate of the Bchl–protein reaction was proportional in this pH region to the fifth power of H^+ concentration. Below pH 1.5 a blue intermediate appeared in the reaction mixture in addition to the pink pheophytin. This led to the conclusion that the reaction consisted of at least two steps: a conformational change in the Bchl–protein to form a blue intermediate (I 790), and the pheophytinization of Bchl in the blue intermediate to form Bph–protein. The dependence of the first reaction on the fifth power of $[H^+]$ and the dependence of the pheophytinization proper on the first power permitted the ratio of the two rates to be altered by proper adjustment of the pH.

In the present work, the reaction was carried on at such high acid concentrations that the conversion of Bchl–protein to I 790 was completed in 1 to 2 sec. Thereafter, the spectrophotometer recorded the conversion of I 790 to Bph–protein. The pheophytinization reaction was thereby partially isolated and some complications associated with conformational changes of the protein were eliminated.

The high acid concentration might have caused hydrolysis of the phytol tails of some Bchl molecules. We did not study this possibility, but the work of SCHANDERL, CHICHESTER AND MARSH[6] and of PAKSHINA AND KRASNOVSKII[1] indicates no great difference between the rates of pheophytinization for the chlorophylls and for their chlorophyllides.

Though the kinetics of the removal of magnesium from various chlorophylls and their analogs in solutions have been studied by many workers, such studies are rare for chlorophylls in other states. WILLSTÄTTER AND STOLL[7] noticed the pheophytinization of colloidal chlorophyll by CO_2. More recently, ROSOFF AND ARON[8] studied the reaction of chlorophyll *a* in monomolecular films on aqueous substrates. Our system offered the opportunity to study the reaction within a protein. Sedimentation studies showed that the pigment is attached to the protein in the end-product of the reaction, Bph–protein (F. W. STUDIER, J. M. OLSON AND A. K. GHOSH, (1966) unpublished work). Thus the removal of magnesium takes place while the pigment molecule is attached to the protein. In this respect, the situation is similar to that of the chlorophylls in chloroplasts and chromatophores, and a study of the reactivity of the Bchl in the complex was thought to have relevance to the question of the stability of chlorophylls *in vivo*.

EXPERIMENTAL

Materials

The isolation and purification of the Bchl–protein have been described[2,3]. Samples were stored in the dark at −5° as concentrates in 0.13 M NaCl–0.01 M phosphate (pH 7.8). Solutions were prepared from these stock solutions by dilution. NaCl and LiCl were reagent grade (Baker). KOH (CO_2 free) and HCl were standardized solutions (Bio-Rad).

Methods

Absorption spectra of stable substances were measured with a recording spectrophotometer (Cary 14R) with 1-cm quartz cells and thermostated cell compartment.

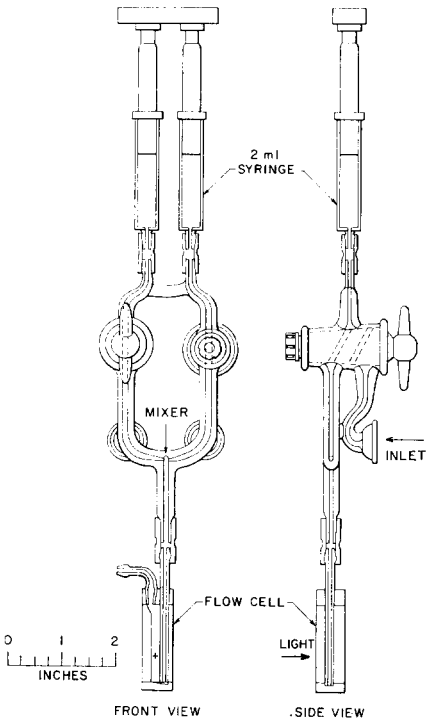


Fig. 1. Apparatus for mixing Bchl–protein and HCl in the spectrophotometer flow cell.

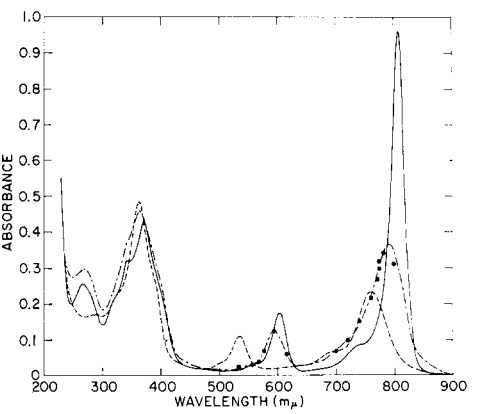


Fig. 2. Absorption spectra of Bchl–protein and its derivatives: ———, Bchl–protein in 0.01 M phosphate (pH 7.2); – – – –, Bph–protein in 0.02 M HCl; –·–·–·–, I 790 in 0.2 M NaCl–0.01 M phosphate (pH 5–6); ●, absorbance values for I 790 from the kinetic data (see text); [Bchl–protein] = 51 mg/l.

For the kinetic studies, a two-jet mixer (Fig. 1) was constructed. Equal volumes (2 ml) of Bchl–protein–LiCl solution and HCl were forced into the mixer chamber and thence into a 1-cm flow cell (Hellma No. 154) placed in the spectrophotometer. The temperature of the cell compartment and the mixer reservoirs was controlled by circulation of constant-temperature water. The response time of the mixer-spectrophotometer system (about 1 sec) was approximately equal to the time required to generate the blue intermediate from the native complex under the experimental conditions employed. Thus the initial reading on the recorder chart was that of I 790.

The experiments were generally carried out under atmospheric conditions. To check whether the oxidation of the isocyclic ring [6,8] or photoreduction[9] could have affected the results, experiments were carried out in an atmosphere of N_2 and in the presence of ascorbic acid (to accentuate any effect of photoreduction, pheophytinization of the photoreduced product, *etc.*). No significant effects were observed.

RESULTS

The blue intermediate I 790

When native Bchl–protein was mixed with HCl to a pH < 1.5, the initial reaction was the denaturation of the protein to form a blue intermediate I 790 in which the Bchl molecules, though still attached to the protein, could be pheophytinized. The intermediate was stabilized by immediate neutralization of the acid and its spectrum determined after correction for the presence of small amounts of pheophytin. In Fig. 2 the absorption spectra of Bchl–protein, I 790 and Bph–protein are shown, for the same molar concentration of the pigments.

An independent determination of the absorbance values of I 790 at several wavelengths was possible from the kinetic data. Since the initial absorbance value (A_0) in the kinetic run was due to I 790 and the final absorbance value (A_∞) due to Bph–protein of equivalent concentration, accurate absorbance values for the intermediate were obtained from the relation, $A_I = A_{ph} \cdot A_0/A_\infty$, for several wavelengths, where A_{ph} is the absorbance of Bph–protein (shown in Fig. 2) obtained from a careful determination of the absorption spectrum. The absorbance values of I 790 obtained by this method are also shown in Fig. 2. The close fit of the spectra obtained by the different methods provides evidence for the correctness of these spectra; it also serves as an internal check for the complete conversion of the Bchl–protein into I 790 in the 1 sec of mixing.

Evidence for a second intermediate I 770

In the region 760–790 mμ, the absorptivity of I 790 is higher at any wavelength than the absorptivity of Bph–protein. As the reaction proceeds, the absorbance decreases with time. At 535 mμ, on the other hand, Bph–protein has an absorption maximum and the absorbance increases with the progress of the reaction. Assuming I 790 and Bph–protein to be the only species spectroscopically identifiable, one can calculate the fraction (x) of I 790 at any time, in either wavelength region from the relation,

$$x = \frac{A_t - A_\infty}{A_0 - A_\infty}$$

where A_t is the absorbance at time t.

In Fig. 3, the apparent course of the reaction in 0.2 M HCl at 25° is shown at several wavelengths. The reaction appears to be faster when the disappearance of I 790 is observed than when the appearance of Bph–protein is followed. This apparent difference in kinetics suggests that a second intermediate is formed from I 790 prior to the formation of Bph–protein.

Additional support for this hypothesis came from observations on the effect of ionic strength (I). In Fig. 4, the effect of a change in I on the course of the reaction in 0.2 M HCl observed at 535 mμ and 783 mμ is shown. On increasing I from 0.2 to 0.5, the reaction is slowed down, but the reduction of the rate is not significant for the first 10 sec. The second feature to be noticed is that the deviation of the 535-mμ curve from the 783-mμ curve increases with increasing I. Both of these observations are consistent with the hypothesis of a transformation of I 790 to a second intermediate and the pheophytinization of Bchl in each intermediate. The transformation is

presumed to be accelerated by an increase in I, whereas the pheophytinization is retarded by an increase in I.

Further support for a second intermediate came from the effect of heating the native Bchl–protein before mixing with acid. Preheating for 80–100 min at 45° increased the initial rate of pheophytinization as shown in Fig. 5. Since it is unlikely for preheating to affect the intrinsic removability of magnesium from Bchl, these results suggest a conformational difference in the initial blue intermediate formed from preheated Bchl–protein. This is consistent with the fluorescence behavior which suggests that the conformation of native Bchl–protein in solution may be temperature dependent (J. M. OLSON AND C. SYBESMA, (1967) unpublished work).

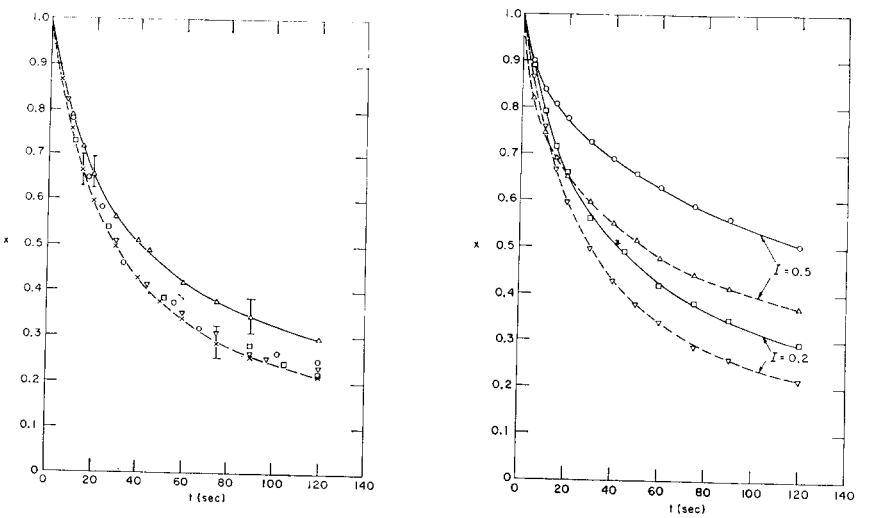


Fig. 3. Conversion of I 790 to Bph–protein in 0.2 M HCl at 25° observed at different wavelengths. △—△, 535 mμ; □, 775 mμ; ×--×, 783 mμ; ▽, 790 mμ; ○, 809 mμ. Estimates of uncertainties are shown on the curves for 535 and 783 mμ. [Bchl–protein] = 49 mg/l. $x = (A_t - A_\infty)/(A_0 - A_\infty)$.

Fig. 4. Variation in the apparent course of the reaction in 0.2 M HCl at 25° on increasing I. Solid curves for 535 mμ and dashed curves for 783 mμ. [Bchl–protein] = 49 mg/l.

It seems that the Bchl molecules in both intermediates are reactive towards H^+. If the Bchl in I 790 were protected and the formation of a second intermediate were necessary for the pheophytinization, a build up of the second intermediate and an induction period before the formation of Bph–protein would have been observed. The rate of appearance of Bph–protein would have also been constant over a considerable range. This was not observed in our experiments; therefore some of the Bchl molecules in both intermediates must be in contact with the solvent.

A very approximate absorption spectrum of the second intermediate was calculated from the apparent course of the reaction observed at various wavelengths, assuming the rate of pheophytinization to be the same in both intermediates and with an arbitrarily adjustable rate of conversion of I 790 into the second intermediate. This spectrum has an absorption maximum in the 770-mμ region. The second intermediate is therefore designated I 770. An intermediate with a similar absorption spectrum has been observed during the alkaline degradation of Bchl–protein[4].

Treatment of the kinetic data

Most of the data were obtained for the same initial concentration (49 mg/l) of Bchl–protein and show similar features under different conditions. A control experiment was run with a more concentrated solution of Bchl–protein, and the same general properties were observed.

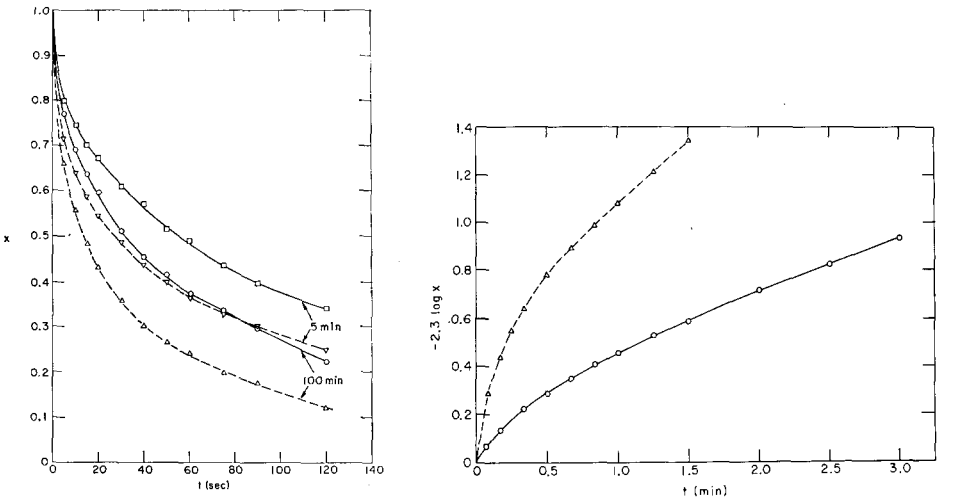


Fig. 5. Effect of 5 and 80–100 min preincubation of Bchl–protein at 45°. Reaction carried out at 45° in 0.2 M HCl (I = 0.5). Solid curves for 535 mμ and dashed curves for 783 mμ. [Bchl–protein] = 49 mg/l.

Fig. 6. First-order plots for the pheophytinization reaction at 23° carried out under two different conditions. O—O, [Bchl–protein] = 0.31 g/l, [H^+] = 0.25 M, I = 0.25, λ = 535 mμ; △--△, [Bchl–protein] = 49 mg/l, [H^+] = 0.4 M, I = 0.4, λ = 783 mμ.

For further treatment of the data, decay curves similar to those presented in Figs. 3–5 were assumed to represent the true course of disappearance of Bchl. This approximation was used to circumvent the difficulty posed by the formation of I 770. Data obtained at 535 mμ and 783 mμ for the same reaction conditions were analysed simultaneously, since the deviations at the two wavelengths were expected to be in opposite directions. Similar results are obtained at both wavelengths, even though the numerical values for the calculated quantities differ somewhat.

In contrast to the kinetics of chlorophyll disappearance observed in organic solvents, a conventional first-order plot for the disappearance of I 790 is non-linear and bends towards the abscissa. In the two reactions plotted in Fig. 6, the experimental parameters are widely different in regard to protein concentration, ionic strength, acid concentration, and the wavelength of observation, but each curve exhibits the typical non-linear character. This non-linearity cannot be explained by the formation of I 770, if the Bchl molecules in I 770 are assumed to be more exposed to H^+ than those in I 790. If all the Bchl molecules in a given intermediate were identical, we would have obtained curves bending away from the abscissa. It seems, therefore, that the 20 Bchl molecules in both intermediates are heterogeneous in reactivity.

The order of the reaction

To understand the mechanism of the reaction better, we treated the rate data by a differential method similar to that employed by CASEY AND LAIDLER[10,11]. To distinguish between [Bchl–protein] and [Bchl] (which is determined experimentally), we present the data as $\dot{x}$ versus x. The equation for the rate of a reaction is conventionally written as $-\dot{c} = kc^n$, where n is defined as the order of the reaction. We, however, used the analogous expression

$$-c_0\dot{x} = kc_0{}^{n_c}x^{n_x} \tag{1}$$

where c_0 is the initial concentration of Bchl–protein. In logarithmic form this gives

$$\log(-\dot{x}) = \log k + (n_c - 1)\log c_0 + n_x \log x \tag{2}$$

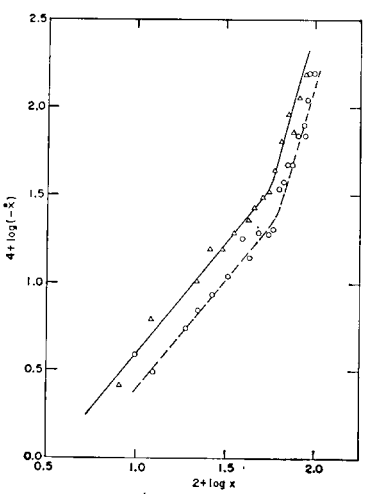
The order with respect to x, n_x, is obtained by plotting $-\dot{x}$ *vs.* $\log x$ and measuring the slopes at different values of x. In Fig. 7, such plots are shown for two values of c_0. As shown in Fig. 8, n_x approaches the value 1 as the reaction nears completion, *i.e.* at low values of x. At the beginning of the reaction, however, the slope is very high with values of n_x approaching 7 and 8.

An estimate of the order with respect to concentration, n_c (the true order of the reaction), can be obtained from the comparison of the two curves in Fig. 7, and the Eqn. 3

$$n_c = \frac{\log(c_1\dot{x}_1/c_2\dot{x}_2)}{\log(c_1x_1/c_2x_2)}, \; x_1 = x_2 \tag{3}$$

where c_1 and c_2 are two initial concentrations and x_1 and x_2 refer to the fraction of Bchl remaining in each case. Calculations for various values of x give values of n_c at various stages of the reaction. In Fig. 8 are plotted the values of n_c calculated from the pair of experiments shown in Fig. 7.

The essential constancy of n_c for all values of x shows that the course of the



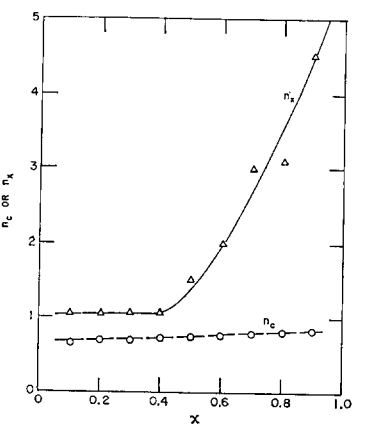


Fig. 7. Log $(-\dot{x})$ *vs.* log x. Reactions at 23° in 0.25 M HCl (I = 0.25) observed at 535 mμ. △—△, [Bchl–protein] = 49 mg/l; ○--○, [Bchl–protein] = 0.31 g/l.

Fig. 8. Variation of n_c and n_x as a function of x. Data from Fig. 7.

reaction is not substantially affected by the protein concentration. The very great variation of n_x, on the other hand, reflects the complexity of the reactions involving any given chlorophyll–protein macromolecule. Since this complexity cannot be attributed to interaction between macromolecules nor to a back-reaction, it can only be due to differences in the reactivity of the 20 Bchl molecules in each macromolecule. Thus we have

$$\text{Bchl}_1 \xrightarrow{k_1} \text{Bph}_1$$
$$\text{Bchl}_2 \xrightarrow{k_2} \text{Bph}_2$$
$$\text{Bchl}_n \xrightarrow{k_n} \text{Bph}_n$$

At the maximum, 20 rate-constants would be necessary to describe the reaction. In practice, five rate-constants should be sufficient, since the 20 Bchl-molecules appear to be grouped in four subunits of the complex[3].

Dependence on acid concentration

The rate of pheophytinization of the intermediates increased with increasing acid concentration. To obtain the quantitative dependence on the acid concentration, the reactions were followed in HCl–LiCl mixtures with I constant at 0.5. Furthermore, to obviate the problems associated with multiple reaction sites, the reaction rates were determined at various stages of conversion. In Fig. 9, the normalized rates $(-\dot{x}/x)$ at 25 %, 40 % and 60 % conversion to Bph–protein are plotted against the acid concentration. Rates at 0 % conversion are not included because of the large errors in the estimation. At a given stage of the reaction, the rate is directly proportional to the acid concentration. However, at each acid concentration the normalized rate falls off as the reaction proceeds. This again reflects the fact that the 20 Bchl molecules in I 790 or I 770 are not all equivalent.

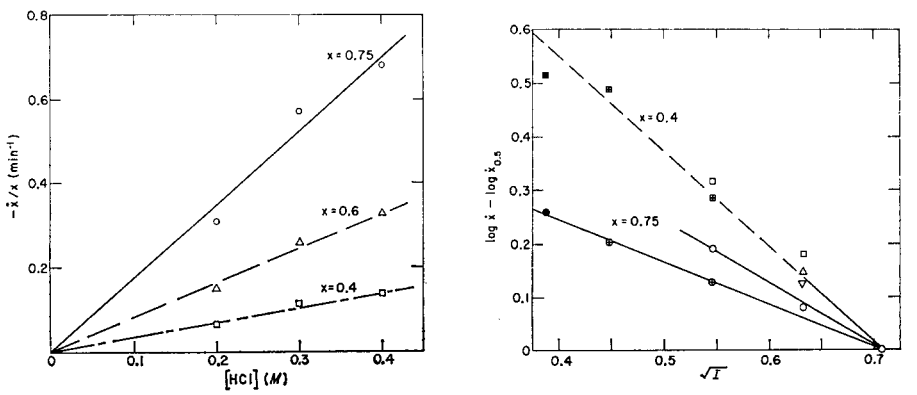


Fig. 9. Normalized rate of pheophytinization $(-\dot{x}/x)$ *vs.* HCl concentration. Rates were measured at 535 mμ for three stages of the reaction (x = 0.75, 0.6, and 0.4) with T = 25° and I = 0.5.

Fig. 10. Dependence of reaction rate (783 mμ) on I. The values of [HCl] are indicated by the various symbols as follows. For x = 0.4: ■, 0.15 M; ⊞, 0.20 M; □, 0.30 M; ▽, 0.4 M. For x = 0.75: ●, 0.15 M; ⊕, 0.20 M; ○, 0.30 M; △, 0.4 M. The slopes of the three lines are −0.80, −1.18, and −1.82, respectively. The solid lines are for x = 0.75; the dashed line for x = 0.4. See text for details.

Effect of ionic strength

The reaction was studied at various concentrations of LiCl to observe the effect of ionic strength. As shown in Fig. 4 the general effect of increasing I is to decrease the reaction rate. The reaction rates measured at 783 mμ are summarized in Table I. In Fig. 10 the logarithm of the ratio of $-\dot{x}$ to $-\dot{x}_{0.5}$ for several values of [HCl] is plotted *vs.* $\sqrt{I}$ because of an apparent linear dependence of log $(-\dot{x})$ on $\sqrt{I}$. (The rates for $I = 0.5$ are designated $-\dot{x}_{0.5}$). The rate ratio $\dot{x}/\dot{x}_{0.5}$ is dependent on the acid concentration early in the reaction (*i.e.*, when $x = 0.75$), but becomes essentially

TABLE I

REACTION RATES

The normalized reaction rates $(-\dot{x}/x)$ in min^{-1} measured at 783 mμ for various values of HCl concentration and I

HCl (*M*)	I	*Normalized reaction rate* (min^{-1})		
		x: 0.75	0.60	0.40
0.15	0.15	1.12	0.76	0.44
	0.50	0.61	0.35	0.13
0.20	0.20	1.65	1.30	0.75
	0.30	1.40	0.84	0.46
	0.50	1.04	0.48	0.24
0.30	0.30	1.80	1.24	0.72
	0.40	1.40	0.92	0.52
	0.50	1.16	0.64	0.34
0.40	0.40	2.47	1.43	0.69
	0.50	1.76	1.10	0.52
0.50	0.50	3.2 (approx.)	1.9 (approx.)	0.82 (approx.)

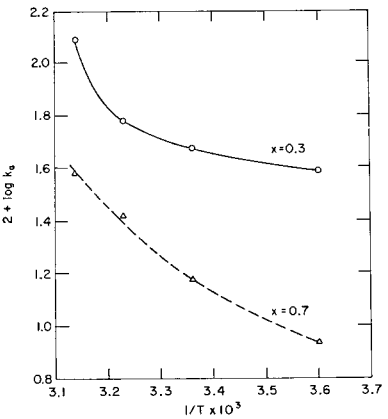
independent of acid concentration later in the reaction (*i.e.*, when $x = 0.4$). This is illustrated in Fig. 10 by the dashed line ($x = 0.4$) which fits the empirical equation, $\log (\dot{x}/\dot{x}_{0.5}) = 1.3 - 1.8 \cdot \sqrt{I}$. One solid line drawn to fit the datum points for $x = 0.75$ and [HCl] = 0.15 M or 0.20 M has a slope of −0.8, and shows that the net rate of change at 783 mμ early in the reaction is less dependent on I than the rate later on in the reaction. The solid line fitted to the datum points for $x = 0.75$ and [HCl] = 0.3 M shows a negative increase in slope to −1.2. If, finally, the acid concentration is raised to 0.4 M, the slope increases negatively to −1.8, and the dependence on I is the same throughout the reaction ($0.4 \leqslant x \leqslant 0.75$).

The decrease in the rate of pheophytinization on addition of electrolytes is significant because the rate of removal of magnesium from Bchl should not be affected by I in view of the non-ionic nature of Bchl. The charge repulsion between the H^+ and the charge on the protein intermediate should be relieved at higher concentrations of electrolyte, leading to a faster reaction. A possible explanation for the observed reduction in the rate is that the collapse of the extended charged protein at high salt concentrations decreases the accessibility of the Bchl molecules to H^+.

Effect of temperature

Experiments in 0.2 M HCl ($I = 0.5$) were conducted at 5°, 25°, 35°, and 45°, in an attempt to determine the energy of activation, $\Delta E^{\ddagger}$, of the pheophytinization

process in the protein. Unlike the straight line plots of ln k *vs.* $1/T$ obtained by SCHANDERL, CHICHESTER AND MARSH[6] for chlorophylls *a* and *b* and their derivatives, similar plots for Bchl complexed with protein were complicated by the variation in rate as the reaction progressed. Large deviations from linearity were exhibited when the logarithm of the apparent first-order rate-constant k_a, at a particular value of the fractional conversion $(1-x)$, was plotted against $1/T$ (Fig. 11) (*cf.*



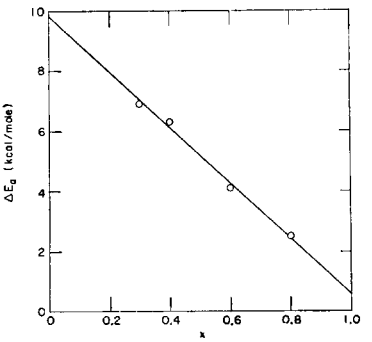


Fig. 11. Log k_a *vs.* $1/T$ for the pheophytinization reaction in 0.2 M HCl ($I = 0.5$) at $x = 0.7$ and 0.3.

Fig. 12. Apparent activation energy ($\Delta E_a^{\ddagger}$) as a function of x. The datum for $x = 0.3$ is from Fig. 11.

MACKINNEY AND JOSLYN[12]). The deviations increased at lower values of x. Apparent activation energies ($\Delta E_a^{\ddagger}$) for the range 5° to 35° were calculated from the average slopes of curves like those in Fig. 11, and the values plotted in Fig. 12 as a function of x. Such apparent activation energies depend on more factors than only the change in the rate of removal of magnesium from Bchl. In the early part of the reaction $\Delta E_a^{\ddagger}$ is low; but at 70 % conversion ($x = 0.3$) $\Delta E_a^{\ddagger}$ reaches an approximate value of 7 kcal/mole Bchl, which is close to the value (approx. 10 kcal/mole) required for pheophytinization of chlorophylls *a* and *b* in solution[6].

DISCUSSION

In these experiments, two reactions are contributing simultaneously to the observed absorption changes. The native Bchl–protein complex certainly becomes highly charged by the addition of H^+ at the high acid concentrations and this, presumably, is the cause of the initial rapid denaturation to produce I 790. In contrast to native Bchl–protein, at least some of the Bchl molecules in I 790 are in contact with the solvent as shown by the pheophytinization following denaturation. Changes in the absorption spectrum (λ_{max} shifted from 809 mμ to 790 mμ; ε_{max} reduced by a factor of 2.6), indicate that the environment of the pigment molecules has changed radically. Even in I 790, however, there are some pigment–pigment and/or protein–pigment interactions affecting the absorption spectrum. This is shown by the transformation of I 790 to a second intermediate I 770, the spectrum of which is similar to that of Bchl dissolved in methanol. There may even exist several intermediates

between I 790 and I 770, corresponding to progressive unfolding of the protein and consequent increase of solvent–pigment interaction.

The pheophytinization reactions are superimposed on these conformational changes. H^+ remove magnesium from Bchl molecules in both I 790 and I 770. However, even in these denatured proteins, some of the Bchl molecules appear to be more deeply embedded in the protein than others. (The broadening of the 790- and 770-mμ bands in the intermediates reflects heterogeneity of the Bchl molecules.) This might explain the heterogeneity in the reaction rates observed. Due to charge repulsion, the concentration of H^+ would fall off from the surface towards the center of the partially denatured protein–chlorophyll complex. The further away from the surface a Bchl molecule were, the lower would be the apparent rate-constant of pheophytinization. About half of the Bchl molecules would seem to be in such a region of low $[H^+]$ from the relatively uniform reaction rate observed after 60 % conversion. Effects of changes in I on the reaction rates give support to this picture. At low I values, the highly charged protein molecule would be more extended than at high I values, and the chlorophyll groups would be more easily attacked. This is one possible reason for the decrease in rate at higher I, in spite of the assistance to H^+ by the additional electrolyte to overcome charge repulsion posed by the highly charged protein. Occupation of sites by Li^+ competing with H^+ may be another factor. Although the actual pheophytinization step is retarded by increasing I, the initial conversion of Bchl protein to I 790 is clearly accelerated by increasing I (ref. 4). From the data in Fig. 10 it appears that the conversion of I 790 to I 770 may also be accelerated by increasing I. This would be consistent with the more negative slope for $x = 0.4$ than for $x = 0.75$. When the observation wavelength is 783 mμ, both the conversion of I 790 to I 770 and the formation of Bph–protein affect the kinetics of the absorbance change. Data obtained with 0.2 M HCl at 535 mμ (not shown), are about the same for $x = 0.4$ and $x = 0.75$, over the range of I values studied. This is consistent with the fact that the formation of Bph–protein is essentially the only reaction observed at 535 mμ.

Useful comparisons can be made with the observations of PAKSHINA AND KRASNOVSKII[1] in acetone–water (9:1, v/v). From their work, the calculated first-order rate-constants for 0.15 M and 0.30 M HCl are 0.55 min^{-1} and 1.1 min^{-1} respectively, if the rate-constant were directly proportional to $[H^+]$. This compares favorably with the extrapolated first-order rate-constants, 1.2 min^{-1} and 2.4 min^{-1} obtained in our experiments at the onset of the reaction, especially when the contribution to our values by more than one reacting species is considered. Therefore, the Bchl molecules which react in the first few seconds have approximately twice the reaction rates of Bchl molecules dissolved in acetone–water. This again indicates the location of some molecules near the surface of the protein in I 790 and I 770. The rate-constants drop about 88 % after 75 % conversion (Fig. 6). Assuming the same intrinsic reactivity of all Bchl molecules, this represents a drop in the real pH in the vicinity of the pigments of about 0.9 unit and again suggests some retention of the structure of the protein.

An explanation for the increase in apparent activation energy as the reaction progresses is difficult to formulate. At low values of x, $\Delta E_a^{\ddagger}$ approaches a value which may reflect direct attack of the remaining Bchl molecules by H^+ in the solvent. The slow rate of pheophytinization at low values of x might be due either to a drop of real pH at the sites of the remaining Bchl molecules or to a rate-limiting step in unfolding the protein core. The low values of $\Delta E_a^{\ddagger}$ obtained for the early part of the

reaction suggest the possibility of direct protonation of some Bchl molecules by charged amino groups in the intermediate(s) produced by acidification.

Some aspects of the present work are pertinent to the question of stability and reactivity of chlorophyll in chloroplasts and chromatophores. As long as the pigment is not exposed to the solvent (*e.g.* in native Bchl–protein), no reaction takes place[4]. Once exposed, however, the chlorophylls react, subject to modification by binding to protein and the location of the pigment in the protein.

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